

# Maternal antibody inhibition of recombinant Newcastle disease virus vectored vaccine in a primary or booster avian influenza vaccination program of broiler chickens

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## ABSTRACT

Maternally-derived antibodies (MDA) provide early protection from disease, but may interfere with active immunity in young chicks. In highly pathogenic avian influenza virus (HPAIV)-enzootic countries, broiler chickens typically have MDA to Newcastle disease virus (NDV) and H5 HPAIV, and their impact on active immunity from recombinant vectored vaccines is unclear. We assessed the effectiveness of a spray-applied recombinant NDV vaccine with H5 AIV insert (rNDV-H5) and a recombinant turkey herpesvirus (HVT) vaccine with H5 AIV insert (rHVT-H5) in commercial broilers with MDA to NDV alone (MDA:AIV<sup>-</sup>NDV<sup>+</sup>) or to NDV plus AIV (MDA:AIV<sup>+</sup>NDV<sup>+</sup>) to provide protection against homologous HPAIV challenge. In Experiment 1, chicks were spray-vaccinated with rNDV-H5 at 3 weeks (3w) and challenged at 5 weeks (5w). All sham-vaccinated progeny lacked AIV antibodies and died following challenge. In rNDV-H5 vaccine groups, AIV and NDV MDA had completely declined to non-detectable levels by vaccination, enabling rNDV-H5 spray vaccine to elicit a protective AIV antibody response by 5w, with 70–78% survival and significant reduction of virus shedding compared to shams. In Experiment 2, progeny were vaccinated with rHVT-H5 and rNDV-H5 at 1 day (1d) or 3w and challenged at 5w. All sham-vaccinated progeny lacked AIV antibodies and died following challenge. In rHVT-H5(1d) vaccine groups, irrespective of rNDV-H5(3w) boost, AIV antibodies reached protective levels pre-challenge, as all progeny survived and virus shedding significantly decreased compared to shams. In contrast, rNDV-H5-vaccinated progeny had AIV and/or NDV MDA at the time of vaccination (1d and/or 3w) and failed to develop a protective immune response by 5w, resulting in 100% mortality after challenge. Our results demonstrate that MDA to AIV had minimal impact on the effectiveness of rHVT-H5, but MDA to AIV and/or NDV at the time of vaccination can prevent development of protective immunity from a primary or booster rNDV-H5 vaccine.

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**Abbreviations:** APMV-1, avian paramyxovirus 1; BHI, brain heart infusion; d, day old; dpc, days post-challenge; dpv, days post-vaccination; EID<sub>50</sub>, mean egg infectious doses; GMT, geometric mean titers; HA, hemagglutinin; HI, hemagglutination inhibition; HPAIV, highly pathogenic avian influenza virus; HVT, turkey herpesvirus; IBDV, infectious bursal disease virus; LPAIV, low pathogenicity avian influenza virus; MDA, maternally-derived antibodies; MDT, mean death time; MDV, Marek's disease virus; NDV, Newcastle disease virus; qRRT-PCR, quantitative real-time reverse transcriptase polymerase chain reaction; rgH5N1, reverse genetics H5N1 vaccine; rHVT-H5, recombinant HVT vaccine with H5 AIV insert; rNDV-H5, recombinant NDV vaccine with H5 AIV insert; SEPRL, Southeast Poultry Research Laboratory; SPF, specific pathogen free; Tk/MN/15, A/turkey/Minnesota/12582/2015 (H5N2) HPAIV; w, weeks old.

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## 1. Introduction

Outbreaks of highly pathogenic (HP) avian influenza (AI) virus (AIV) in poultry and wild birds have had a devastating economic and social impact worldwide [1,2]. The Eurasian H5N1 HPAIV that emerged in late 1990s in China [3] has expanded from Asia to Europe, Africa, and North America [4]. Also, H5 or H7 HPAIV have become enzootic in China, Indonesia, Vietnam, Bangladesh, Hong Kong, Egypt, and Mexico [5]. Newcastle disease (ND) is a significant worldwide disease of poultry caused by virulent strains of avian avulavirus 1 (former avian paramyxovirus 1 [APMV-1]), commonly known as Newcastle disease virus (NDV) [6–8]. The NDV is enzootic in multiple countries in Europe, Africa, the Middle East, Asia, Central America, and the northern part of South America, and has resulted in at least 4 panzootic outbreaks since it was first identified in the 1920s [9]. Oncogenic Marek's disease virus (MDV) is a worldwide, highly contagious, lymphoproliferative disease of chickens [10,11]. Therefore, vaccination programs have been developed to control all three pathogens. Routine vaccination against HPAIV has been used in control programs of enzootic countries, generally with inactivated whole-virus vaccines or recombinant vector vaccines expressing the hemagglutinin (HA) protein (i.e. the critical antigen to elicit neutralizing antibodies) with even more countries using targeted or risk-based strategies to reduce the costs and increase the efficiency of the HPAIV vaccination programs [5]. By contrast, routine vaccination against NDV is performed virtually worldwide [12,13], and immunization using MDV serotype 3 (MDV-3), also known as turkey herpesvirus (HVT), is used worldwide to protect chicken populations against MDV, but also HVT is used as a vaccine vector for other important viral poultry diseases including H5 AIV [11].

As a consequence of these routine vaccination campaigns, NDV and/or H5 HA maternally-derived antibodies (MDA) are found in the progeny of vaccinated meat chicken breeder flocks [14–17]. Noteworthy, cell-associated HVT vaccines, the most common type of HVT vaccine preparation, induce protection through cell-mediated immunity, which is not passed through the egg yolk to progeny [10,11]. For AIV, NDV, and other agents, the MDA are naturally passed from the hen to the chick through the egg yolk [18,19]. The type and amount of MDA transferred is representative of the circulating antibodies in the hen (produced from vaccination or by natural infection) at the time the egg was laid, and they have a characteristic half-life similar to host antibodies before they naturally degrade in the chick, usually between 2 and 3 weeks of age [19]. Although MDA can prevent or reduce clinical disease by passive immunization during the first weeks of the chick's life [20,21], they can also hinder the immune response to vaccination as seen with infectious bursal disease virus (IBDV) [22], NDV [16,23,24], and AIV [17,25–31] vaccines. Such MDA interference seems to be one of the reasons for the lack of virus eradication success in several HPAIV-enzootic countries using AIV vaccination, such as Egypt and Mexico [25,27,29,32]. This is particularly relevant for inactivated antigens (which comprise the most widely used field vaccines [33]), that are processed through the exogenous antigen presentation pathway [27,34] and therefore are susceptible to be bound by MDA, preventing proper antigen presentation to B cells and initiation of a primary humoral immune response [34]. Similarly, some recombinant vector vaccines, such as fowlpox or NDV, expressing the HA protein have shown to be impacted by MDA interference not only with the response to the HA protein, but also with the replication of the vector, diminishing the protective immune response to both [35,36].

The prime-boost approach is an effective vaccination strategy in HPAIV control; the viral vector vaccines work best as a primer *in ovo* or at 1 day old at the hatchery, and a different type of vaccine, often an inactivated adjuvanted vaccine, is given later as a boost on

the farm at 3 weeks of age or older [36]. However, inactivated vaccines are negatively impacted by MDA, and their use requires handling and injection of individual chickens on the farm, creating a compromised biosecurity situation and high cost application scenario. As a consequence, there is growing interest for new vaccines and vaccination programs using recombinant vector vaccines that can fight off multiple diseases at the same time, overcome MDA interference, and be mass-applied in the hatchery or on the farm. The recombinant HVT vaccine with H5 AIV insert (rHVT-H5) is designed primarily for subcutaneous administration at 1 day of age in chicks and, because the virus spreads primarily cell to cell, it appears to lack or have minimal suppression when H5 MDA are present [36]. Studies using specific pathogen free (SPF) layers [37,38], commercial broilers [39,40], and commercial layers [41] suggested that rHVT-H5 vaccine is able to confer good protection against different H5N1 HPAIV isolates and clades, and that it is able to overcome the neutralizing effect of H5 MDA. In contrast, the recombinant NDV vaccines with H5 AIV insert (rNDV-H5) can be mass administered by drinking water or aerosol (spray) application. Because the cost of administration is such a large part of the cost of vaccination, a mass vaccination approach is greatly desired and is one of the primary benefits of rNDV-H5 [36]. The rNDV-H5 vaccines have shown to provide protection against LPAIV, HPAIV, and NDV velogenic challenges in SPF chickens without maternal immunity vaccinated by several different routes [36,42]. On the contrary, numerous studies indicate that high levels of NDV and/or H5 MDA can interfere with the protection of the rNDV-H5 vaccine against HPAIV challenge [28,31,36,43]. Yet, some studies using passively-transferred AIV antibody in young layer chicks show that the rNDV-H5 vaccine could provide an initial priming of the immune response [28,31]. Also, a high dose of rNDV-H5 vaccine given by eye drop to 8-day-old broilers seems to overcome AIV and NDV MDA [43].

Despite possible MDA interference to the vector, numerous advantages make rNDV-H5 vaccines ideal for AIV vaccine development [33]: (i) vaccination of chickens for NDV is routine worldwide; (ii) rNDV-H5 vector vaccines can be mass applied through spray in the hatchery or drinking water; (iii) NDV efficiently replicates in AIV-target tissues and organs, thus inducing strong local and systemic immune responses at the respiratory tract [44]; and (iv) NDV replicates in both chickens and turkeys. Overall, these benefits underscore the need for continued evaluation and optimization of rNDV-H5 vaccines and vaccination programs that can overcome passive immunity and be mass-applied in the field. Therefore, the goal of the present study was not to assess the efficacy of rNDV-H5 and rHVT-H5 vaccines for licensing, as both vaccines are registered in multiple countries including China and Mexico [45], but to determine their effectiveness under conditions experienced in a field vaccination program. This study assessed the effectiveness of a spray-applied rNDV-H5 vector vaccine (Experiment 1) and prime-boost protocols using rHVT-H5 and rNDV-H5 vaccines (Experiment 2) in vaccination programs utilizing commercial broiler chickens with MDA for protection against a homologous HPAIV challenge.

## 2. Materials and methods

### 2.1. Vaccines

Four vaccines were utilized in this study. First, a commercial tetravalent inactivated vaccine (hereafter LaSota) (Bursa Guard N-B-R, Boehringer Ingelheim, Gainesville, GA) included LaSota NDV strain, IBDV (standard and variant E strains), infectious bronchitis virus (Massachusetts and Arkansas serotypes), and reovirus (1133, 2408, and MSB strains). The inactivated LaSota vaccine

was administered intramuscularly in the broiler breeders at pre-inactivation titers equivalent to  $7.7 \log_{10}$  PFU/0.5 ml per bird, as per manufacturer's recommendation, to boost pre-existing NDV antibody titers. Second, an experimental inactivated reverse genetics H5N1 vaccine (hereafter rgH5N1) contained the HA gene from clade 2.3.4.4 A/gyrfalcon/Washington/40188-6/2014 (H5N8) HPAIV, with the polybasic cleavage site of the HA gene altered to a typical cleavage site sequence of low pathogenicity (LP) AIV, and the remaining 7 backbone segments from the A/Puerto Rico/8/1934 (H1N1) common vaccine strain. The rgH5N1 virus was inactivated with 0.1%  $\beta$ -propiolactone (Sigma Aldrich, St. Louis, MO) and used to prepare an oil-in-water vaccine utilizing a mineral oil-based emulsion (Montanide ISA 70VG, SEPPIC, Paris, France) [46–49]. The vaccine was administered subcutaneously in half of the broiler breeders in a dose of 512 HA units/0.5 ml per bird to induce H5 AIV humoral antibodies. Third, an experimental rNDV-H5 vector vaccine (hereafter rNDV-H5) based on the NDV LaSota vector expressing the H5 ectodomain from clade 2.3.4.4 A/chicken/Iowa/04-20/2015 (H5N2) HPAIV with the polybasic cleavage site altered to LPAIV [42] (courtesy of Dr. Garcia-Sastre, Mount Sinai School of Medicine, New York, NY) was used. The vaccine was administered to broiler breeder progeny of Experiments 1 and 2 in a spray cabinet made *ad hoc* (courtesy of David Smith, Boehringer Ingelheim) using 40  $\mu$ m nozzle, 50 lb pressure, and 15 min contact time, in a dose of  $7 \log_{10}$  mean egg infectious doses (EID<sub>50</sub>)/ml spray. The rNDV-H5 spray vaccination was validated in a pilot study using SPF White Leghorn chickens ( $n = 5$ ); all chickens shed NDV by the oropharynx during the first 6 days post-vaccination (dpv) (peak virus shedding of  $5 \log_{10}$  EID<sub>50</sub>/ml at 5 dpv) and seroconverted by 9 dpv ( $5.8 \log_2$  geometric mean titers [GMT]) (data not shown). Fourth, an experimental rHVT-H5 vector vaccine (hereafter rHVT-H5) (Boehringer Ingelheim) based on the recombinant HVT vector expressing a codon-optimized synthetic H5 from clade 2.3.4.4 A/chicken/Washington/61-9/2014 (H5N2) HPAIV with the polybasic cleavage site altered to LPAIV was used. The vaccine was administered subcutaneously in broiler breeder progeny of Experiment 2 in a dose of  $3.1 \log_{10}$  PFU/0.2 ml per bird.

## 2.2. Virus

The Eurasian-origin clade 2.3.4.4 A/turkey/Minnesota/12582/2015 (H5N2) HPAIV (Tk/MN/15) isolate was used as challenge virus. The Tk/MN/15 virus was selected because it is chicken-adapted and is representative of the Midwest U.S. H5N2 outbreak (2015) viruses that clustered both phenotypically [50] and phylogenetically [51]. The virus was propagated and titrated by allantoic sac inoculation in 9 day-old embryonating chicken eggs by standard methods [52].

## 2.3. Animals, housing, and experimental design

All procedures were performed according to the requirements of the protocol approved by the Institutional Laboratory Animal Care and Use Committee. Forty broiler breeder hens and 4 roosters (Ross) at 29 weeks of age for Experiment 1, and 48 broiler breeder hens and 6 roosters (Cobb) at 26 weeks of age for Experiment 2, were obtained from commercial producers (courtesy of John Smith and Sarah Tilley, Fieldale Farms Corp., Baldwin, GA). All birds had received a routine field vaccination program that included *in ovo* cell-associated HVT vaccination and multiple post-hatch live NDV vaccinations. All birds were kept at the Southeast Poultry Research Laboratory (SEPR) animal biosafety level 2 (ABSL-2) facilities with the precision feeding regime outlined by the producer and *ad libitum* access to water and 16 h of daily light. In order to reproduce levels of NDV antibody titers in commercial broilers of HPAIV-

and NDV-enzootic countries, all hens received additional doses of tetravalent LaSota vaccine (Fig. 1). For each experiment, half of the hens received 3 doses of rgH5N1 vaccine (Fig. 1) and were used to produce chicks with AIV MDA, in addition to NDV (hereafter MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny). The other half of the hens from each experiment were not vaccinated against AIV (Fig. 1) and used to produce chicks without AIV MDA, but with NDV (hereafter MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny). Two weeks after the last vaccination, serum from hens and yolk from laid eggs were tested by hemagglutination inhibition (HI) test to confirm high levels of AIV and NDV antibodies, as a means to predict the transfer of MDA titers to progeny (Fig. 2). Embryonating eggs were collected and incubated at 37.8 °C (1500 Incubator and 1500 Hatcher, GQF, Savannah, GA) for 21 days. The newly hatched chicks were allocated into different experimental units ( $n = 10$  per group) in ABSL-2 and administered rHVT-H5 vaccine and/or rNDV-H5 vaccine, or were sham-vaccinated according to each experimental design (Tables 1 and 2). At 5 weeks old (5w), all progeny were moved to SEPR ABSL-3 facilities and challenged by the choanal route with  $6.9 \log_{10}$  EID<sub>50</sub> of Tk/MN/15 virus. The inoculum titer was verified by back titration in SPF embryonating chicken eggs.

## 2.4. Sampling

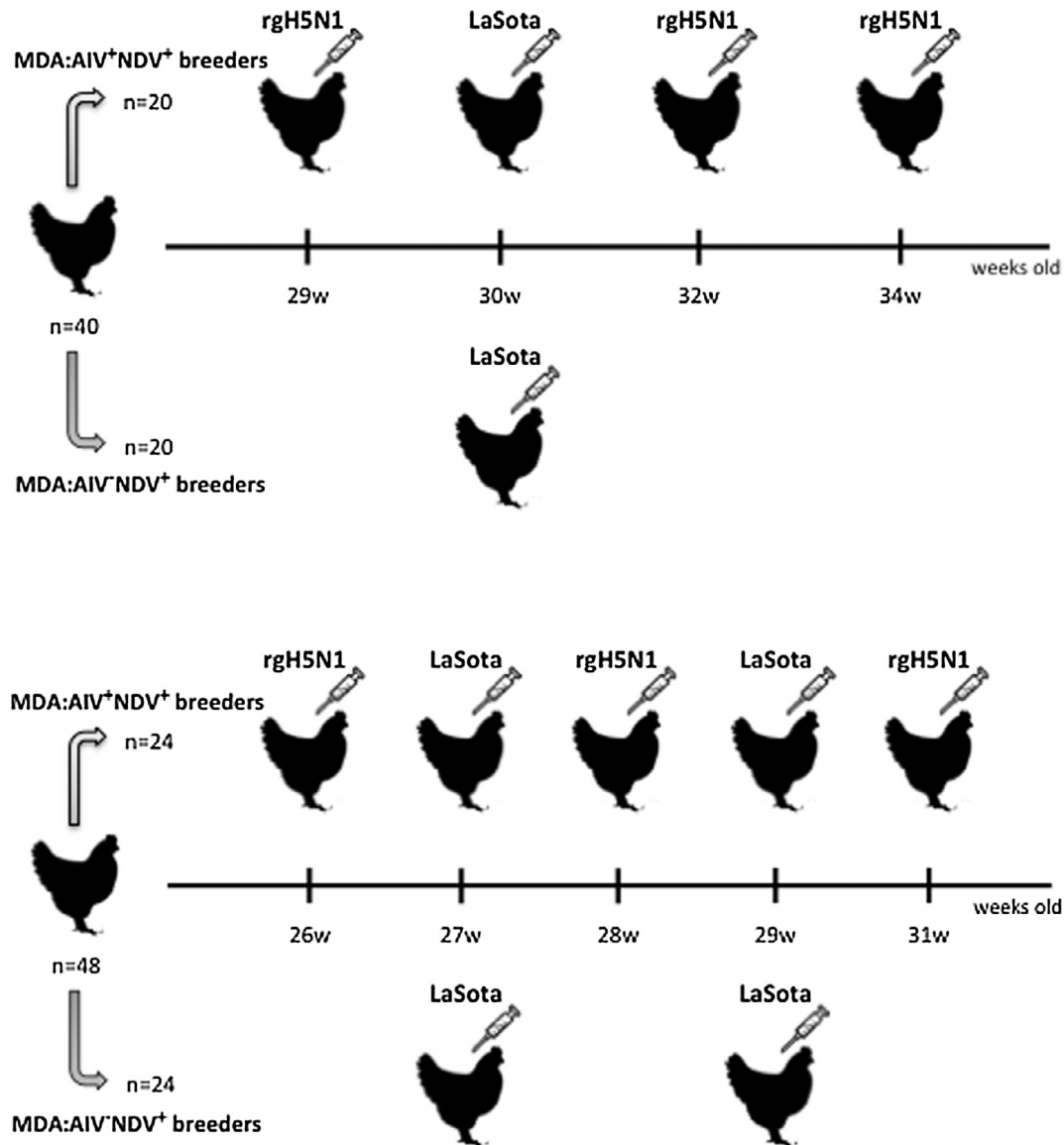
All challenged birds were monitored daily for 2 weeks for clinical signs and mortality. Severely ill birds were euthanized by cervical dislocation and counted as dead for the next day in mean death time (MDT) calculations. Oropharyngeal swabs were collected at 2 and 4 days post-challenge (dpc) in 1.5 ml brain heart infusion (BHI) media (Becton, Dickinson and Company, Sparks, MD) with penicillin (2000 units/ml; Sigma Aldrich), gentamicin (200  $\mu$ g/ml; Sigma Aldrich) and amphotericin B (5  $\mu$ g/ml; Sigma Aldrich). Serum samples were collected at 1 day old (following euthanasia), weekly (1, 2, and 3 weeks old), before challenge (5 weeks old), and at termination (7 weeks old).

## 2.5. Hemagglutination inhibition (HI) assays

Serum and yolk samples were tested by AIV and NDV HI assays. Yolk samples were prepared as previously described [53]. Briefly, yolk material was collected from each test egg and diluted 1:2 in phosphate-buffered saline (Invitrogen, Carlsbad, CA). The mixture was vortexed, incubated at room temperature for 1 h, and centrifuged at  $1500 \times g$  for 30 min at 4 °C. The aqueous phase was collected and used in the HI assay [53]. The HI assays were carried out using antigens clade 2.3.4.4 A/gyrfalcon/Washington/40188-6/2014 (H5N8) HPAIV and LaSota NDV. The antigens were prepared as previously described [54] and the HI assays were performed according to standard procedures [53]. Titers were expressed as  $\log_2$  GMT. Samples with titers below  $3 \log_2$  GMT were considered negative.

## 2.6. Determination of virus shedding from swabs

Swab samples in BHI were processed for quantitative real-time reverse transcriptase polymerase chain reaction (qRRT-PCR) [55] with modifications [56] to determine viral RNA titers. The standard curves for viral RNA quantification were established with RNA extracted from dilutions of the same titrated stocks of the challenge virus. The limit of detection was  $2.0 \log_{10}$  EID<sub>50</sub>/ml; for statistical purposes, qRRT-PCR negative samples were treated as  $1.9 \log_{10}$  EID<sub>50</sub>/ml.



**Fig. 1.** Vaccination schedule for broiler breeders used to obtain experimental progeny. a. Experiment 1 and b. Experiment 2. MDA:AIV<sup>-</sup>NDV<sup>+</sup> breeders, hens that received only NDV vaccinations and produced passively-immunized chicks without AIV MDA, but with NDV MDA (MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny); MDA:AIV<sup>+</sup>NDV<sup>+</sup> breeders, hens that received AIV and NDV vaccinations and produced passively-immunized chicks with AIV and NDV MDA (MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny). The rgH5N1 vaccine was administered subcutaneously at 512 HA units/0.5 ml; inactivated LaSota vaccine was administered intramuscularly at pre-inactivation titers equivalent to 7.7 log<sub>10</sub> PFU/0.5 ml.

## 2.7. Statistical analysis

Mortality and number of birds shedding or seroconverting were tested for statistical significance with Fisher's exact test. Significant difference for mean viral titers in swab samples between groups was analyzed using Kruskal-Wallis test or Mann-Whitney test (GraphPad Prism™ Version 5 software). A p-value of <0.05 was considered to be significant.

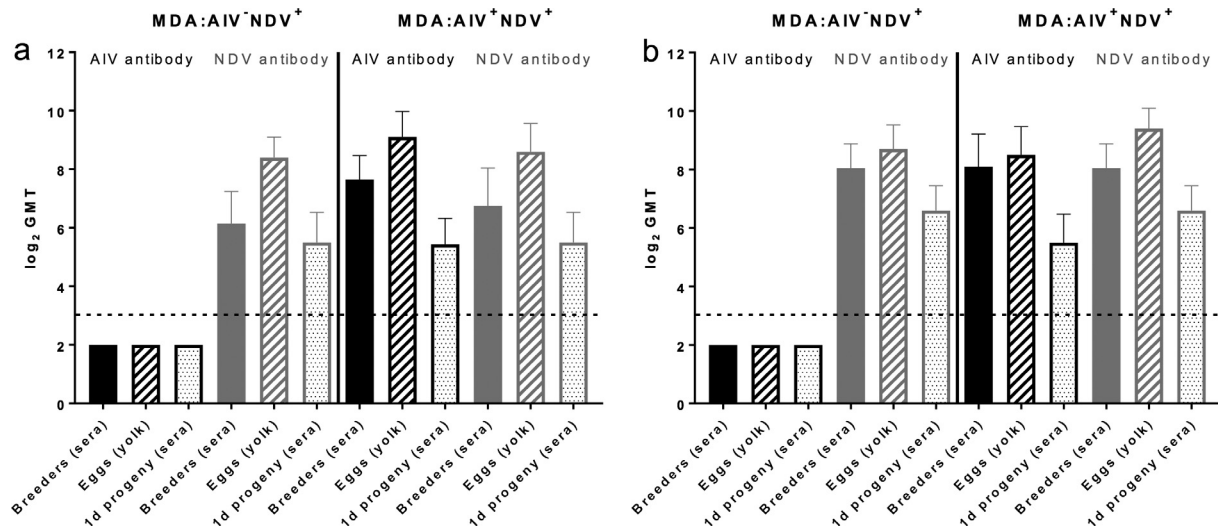
## 3. Results

### 3.1. Experiment 1. Effectiveness of rNDV-H5 live spray vaccination at 3 weeks old in progeny with MDA

**Clinical protection.** After challenge with HPAIV at 5w, 100% of the sham-vaccinated progeny showed acute severe clinical disease and died irrespective of AIV<sup>-</sup> or AIV<sup>+</sup> MDA group, although MDT was slightly longer for MDA:AIV<sup>+</sup>NDV<sup>+</sup> (2.8 days) than

MDA:AIV<sup>-</sup>NDV<sup>+</sup> (2.1 days) sham progeny (Fig. 3, Table 1). The sprayed rNDV-H5(3w) vaccine conferred 78% and 70% clinical protection from HPAIV challenge in MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny, respectively (statistically not different between both groups). The MDT of vaccinated birds that died was slightly longer for MDA:AIV<sup>-</sup>NDV<sup>+</sup> (9.5 days) than MDA:AIV<sup>+</sup>NDV<sup>+</sup> (5 days) progeny, but not statistically different (Fig. 3, Table 1).

**Virus shedding.** Sham-vaccinated progeny shed high HPAIV titers in the oropharynx at 2 dpc (mean titers 6.8 and 6.9 log<sub>10</sub> EID<sub>50</sub>/ml) (Fig. 4, Table 1). The mean HPAIV oropharyngeal titers for MDA:AIV<sup>-</sup>NDV<sup>+</sup> (mean titers 4.3 log<sub>10</sub> EID<sub>50</sub>/ml) and MDA:AIV<sup>+</sup>NDV<sup>+</sup> (mean titers 4.4 log<sub>10</sub> EID<sub>50</sub>/ml) progeny at 2 dpc were significantly lower than their respective sham-vaccinated progeny ( $P \leq 0.0001$ ) and were not statistically different from each other (Fig. 4, Table 1). Similar virus titers were shed at 4 dpc, but the lack of adequate numbers of sham-vaccinated progeny prevented statistical evaluations (Fig. 4).



**Fig. 2.** Antibody titers of broiler breeders and progeny. HI titers for AIV and NDV antibodies in serum from hens, yolk from laid eggs, and serum from 1-day-old progeny of a. Experiment 1 and b. Experiment 2. Titers are expressed as  $\log_2$  GMT. Samples with titers below 3  $\log_2$  GMT were considered negative. MDA:AIV<sup>-</sup>NDV<sup>+</sup> breeders, hens that received only NDV vaccinations and produced passively-immunized chicks without AIV MDA, but with NDV MDA (MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny); MDA:AIV<sup>+</sup>NDV<sup>+</sup> breeders, hens that received AIV and NDV vaccinations and produced passively-immunized chicks with AIV and NDV MDA (MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny).

**Table 1**

Summary of Experiment 1. Progeny were spray-vaccinated with rNDV-H5 at 3 weeks of age and challenged at 5 weeks of age with 6.9  $\log_{10}$  EID<sub>50</sub>/0.1 ml of homologous H5N2 clade 2.3.4.4 HPAIV.

MDA status	Vaccines <sup>1</sup> (age <sup>2</sup> )	Survival (MDT) <sup>3</sup>	Oropharyngeal shedding (2 dpc) <sup>4</sup>	HI serology pre-challenge (5w <sup>2</sup> ) <sup>5</sup>	
				AIV (clade 2.3.4.4)	NDV (LaSota)
AIV <sup>-</sup> NDV <sup>+</sup>	Sham	0% <sup>a</sup> (2.1)	10/10 <sup>a</sup> (6.9) <sup>A</sup>	0/10 (<3)	0/10 (<3)
AIV <sup>-</sup> NDV <sup>+</sup>	rNDV-H5(3w)	78% <sup>b</sup> (9.5)	10/10 <sup>a</sup> (4.3) <sup>B</sup>	9/9 (3.9)	8/9 (6.1)
AIV <sup>+</sup> NDV <sup>+</sup>	Sham	0% <sup>a</sup> (2.8)	10/10 <sup>a</sup> (6.8) <sup>A</sup>	0/10 (<3)	0/10 (<3)
AIV <sup>+</sup> NDV <sup>+</sup>	rNDV-H5(3w)	70% <sup>b</sup> (5)	10/10 <sup>a</sup> (4.4) <sup>B</sup>	8/10 (4)	10/10 (6.3)

<sup>1</sup> rNDV-H5 = recombinant NDV vaccine with H5 gene insert from clade 2.3.4.4 (7  $\log_{10}$  EID<sub>50</sub>/dose, spray).

<sup>2</sup> 3w = 3 weeks old; 5w = 5 weeks old.

<sup>3</sup> Different superscript lowercase denotes statistically significant differences in survival between progeny groups ( $p < 0.05$ ). In parenthesis, mean death time (MDT) of birds that died.

<sup>4</sup> The numbers represent no. virus positive/total in group followed by mean virus shed titer expressed as  $\log_{10}$  EID<sub>50</sub>/ml. Different superscript lowercase denotes statistical significance of number of birds shedding among groups by Fisher Exact or Chi square tests ( $p < 0.05$ ). Different superscript uppercase denotes statistical significance of shedding titers among groups by Mann-Whitney test ( $p < 0.05$ ).

<sup>5</sup> The numbers represent no. serology positive/total in group followed by mean HI titers against AIV or NDV antigen expressed as  $\log_2$  GMT. Negative titers defined as  $< 3 \log_2$  GMT.

**Table 2**

Summary of Experiment 2. Progeny were vaccinated with rHVT-H5 and rNDV-H5 in different combinations and challenged at 5 weeks of age with 6.9  $\log_{10}$  EID<sub>50</sub>/0.1 ml of homologous H5N2 clade 2.3.4.4 HPAIV.

MDA status	Vaccines <sup>1</sup> (age <sup>2</sup> )	Survival (MDT) <sup>3</sup>	Oropharyngeal shedding (2 dpc) <sup>4</sup>	HI serology pre-challenge (5w <sup>2</sup> ) <sup>5</sup>	
				AIV (clade 2.3.4.4)	NDV (LaSota)
AIV <sup>-</sup> NDV <sup>+</sup>	Sham	0% <sup>a</sup> (2.2)	10/10 <sup>a</sup> (7.2) <sup>A</sup>	0/10 (<3)	0/10 (<3)
AIV <sup>-</sup> NDV <sup>+</sup>	rHVT-H5(1d)	100% <sup>b</sup>	4/10 <sup>b</sup> (2.4) <sup>B</sup>	10/10 (7.6)	0/10 (<3)
AIV <sup>-</sup> NDV <sup>+</sup>	rNDV-H5(1d)	0% <sup>a</sup> (3.4)	10/10 <sup>a</sup> (6.5) <sup>A</sup>	1/10 (3)	0/10 (<3)
AIV <sup>-</sup> NDV <sup>+</sup>	rHVT-H5(1d) + rNDV-H5(3w)	100% <sup>b</sup>	3/10 <sup>b</sup> (2.3) <sup>B</sup>	10/10 (8.2)	10/10 (6.3)
AIV <sup>-</sup> NDV <sup>+</sup>	rNDV-H5(1d) + rNDV-H5(3w)	0% <sup>a</sup> (3.7)	10/10 <sup>a</sup> (5.7) <sup>A</sup>	5/10 (3)	7/10 (4.3)
AIV <sup>+</sup> NDV <sup>+</sup>	Sham	0% <sup>a</sup> (3.6)	10/10 <sup>a</sup> (6.1) <sup>A</sup>	0/10 (<3)	0/10 (<3)
AIV <sup>+</sup> NDV <sup>+</sup>	rHVT-H5(1d)	100% <sup>b</sup>	3/9 <sup>b</sup> (2.5) <sup>B</sup>	9/9 (5.9)	0/9 (<3)
AIV <sup>+</sup> NDV <sup>+</sup>	rNDV-H5(1d)	0% <sup>a</sup> (3.1)	7/8 <sup>a</sup> (5.7) <sup>A</sup>	3/8 (3)	0/8 (<3)
AIV <sup>+</sup> NDV <sup>+</sup>	rHVT-H5(1d) + rNDV-H5(3w)	100% <sup>b</sup>	5/9 <sup>b</sup> (2.3) <sup>B</sup>	10/10 (6.4)	10/10 (6.2)
AIV <sup>+</sup> NDV <sup>+</sup>	rNDV-H5(1d) + rNDV-H5(3w)	0% <sup>a</sup> (3)	10/10 <sup>a</sup> (7.2) <sup>A</sup>	4/10 (3.3)	9/10 (4.1)

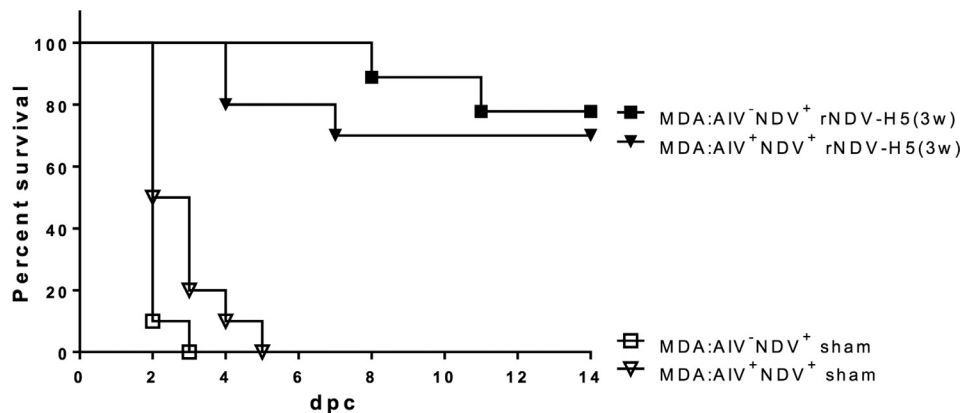
<sup>1</sup> rHVT-H5 = recombinant HVT vaccine with H5 gene insert from clade 2.3.4.4 (3.1  $\log_{10}$  PFU/dose, subcutaneous); rNDV-H5 = recombinant NDV vaccine with H5 gene insert from clade 2.3.4.4 (7  $\log_{10}$  EID<sub>50</sub>/dose, spray).

<sup>2</sup> 1d = 1 day old; 3w = 3 weeks old; 5w = 5 weeks old.

<sup>3</sup> Different superscript lowercase denotes statistically significant differences in survival between progeny groups ( $p < 0.05$ ). In parenthesis, mean death time (MDT) of birds that died.

<sup>4</sup> The numbers represent no. virus positive/total in group followed by mean virus shed titer expressed as  $\log_{10}$  EID<sub>50</sub>/ml. Different superscript lowercase denotes statistical significance of number of birds shedding among groups by Fisher Exact or Chi square tests ( $p < 0.05$ ). Different superscript uppercase denotes statistical significance of shedding titers among groups by Mann-Whitney test ( $p < 0.05$ ).

<sup>5</sup> The numbers represent no. serology positive/total in group followed by mean HI titers against AIV or NDV antigen expressed as  $\log_2$  GMT. Negative titers defined as  $< 3 \log_2$  GMT.



**Fig. 3.** Survival curve of Experiment 1. MDA:AIV<sup>-</sup>NDV<sup>+</sup> rNDV-H5(3w), progeny without AIV MDA but with NDV MDA spray-vaccinated with rNDV-H5 at 3 weeks of age; MDA:AIV<sup>+</sup>NDV<sup>+</sup> rNDV-H5(3w), progeny with AIV and NDV MDA spray-vaccinated with rNDV-H5 at 3 weeks of age; MDA:AIV<sup>-</sup>NDV<sup>+</sup> sham, progeny without AIV MDA but with NDV MDA sham-vaccinated at 3 weeks of age; MDA:AIV<sup>+</sup>NDV<sup>+</sup> sham, progeny with AIV and NDV MDA sham-vaccinated at 3 weeks of age.

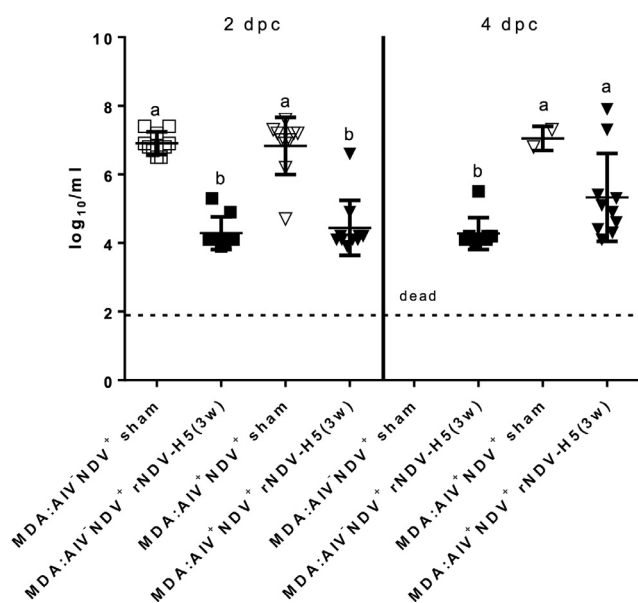
**Serology.** The MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny had high AIV MDA (5.4 log<sub>2</sub> GMT) at 1 day old, which completely declined in all birds below the detectable limit by 3 weeks old (Fig. 5a). Both MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny that received spray rNDV-H5(3w) vaccine seroconverted with group AIV titers of 3.9 and 4 log<sub>2</sub> GMT, respectively, at the time of challenge (pre-challenge or 2 weeks post vaccination) (Fig. 5a, Table 1). These vaccinated birds had an anamnestic response (2.6–4 fold increase) after challenge (statistically not different between MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny) that conferred protection against lethal HPAIV challenge. Interestingly, the 5 vaccinated birds that succumbed to infection had no (<3 log<sub>2</sub> GMT) or low (3–4 log<sub>2</sub> GMT) AIV titers at challenge. In contrast, all sham-vaccinated progeny lacked AIV titers at challenge, either because they never had AIV MDA titers (MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny) or because AIV MDA had

already declined below the detectable limit (MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny) (Fig. 5a, Table 1), and were not protected against HPAIV challenge. All progeny had high NDV MDA (5.5 log<sub>2</sub> GMT) at 1 day old, which completely declined by 3 weeks old (Fig. 5b). Both MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny that received spray rNDV-H5(3w) vaccine seroconverted with group NDV titers of 6.1 and 6.3 log<sub>2</sub> GMT, respectively, at the time of challenge (Fig. 5b, Table 1). These vaccinated birds maintained their NDV titers after challenge, which were not statistically different between both progeny groups (Fig. 5b).

### 3.2. Experiment 2. Effectiveness of prime-boost live vaccination in progeny with MDA

**Clinical protection.** After challenge with HPAIV at 5w, 100% of the sham-vaccinated progeny showed acute severe clinical disease and died irrespective of AIV<sup>-</sup> or AIV<sup>+</sup> MDA group; MDT was slightly longer for MDA:AIV<sup>+</sup>NDV<sup>+</sup> (3.6 days) than MDA:AIV<sup>-</sup>NDV<sup>+</sup> (2.2 days) sham progeny, but not statistically different (Fig. 6, Table 2). Progeny that received spray rNDV-H5(1d) and/or rNDV-H5(3w) vaccine had 100% mortality after HPAIV challenge; MDT was slightly longer for rNDV-H5 vaccinated MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny than corresponding sham-vaccinated progeny (not statistically different), but the same was not observed for MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny. Progeny that received rHVT-H5(1d) vaccine, irrespective of rNDV-H5(3w) boost, had 100% clinical protection against challenge regardless of AIV<sup>-</sup> or AIV<sup>+</sup> MDA group (Fig. 6, Table 2).

**Virus shedding.** Sham-vaccinated MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny shed high titers of HPAIV in oropharynx at 2 dpc (mean titers 6.1 and 7.2 log<sub>10</sub> EID<sub>50</sub>/ml) (Fig. 7, Table 2). Progeny that received spray rNDV-H5(1d) and/or rNDV-H5(3w) vaccine shed similar quantities of virus in oropharynx at 2 dpc as compared to sham-vaccinated progeny, with no statistical difference in the numbers of birds shedding virus nor the titers of virus shed. Similar virus titers were shed at 4 dpc, but the lack of adequate numbers of sham-vaccinated progeny prevented statistical evaluations (Fig. 7). In contrast, MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny that received rHVT-H5(1d) vaccine, irrespective of rNDV-H5(3w) boost, had a significant decrease in both shedding titers and numbers of birds shedding virus at 2 dpc as compared to corresponding sham-vaccinated progeny (Fig. 7, Table 2). Virus titers shed by these vaccinated birds at 4 dpc remained statistically not different from titers shed at 2 dpc (Fig. 7).



**Fig. 4.** Scatter plot of oropharyngeal shedding from progeny of Experiment 1. Shedding titers are expressed as log<sub>10</sub> with error bars. The limit of detection was 2.0 log<sub>10</sub> EID<sub>50</sub>/ml; for statistical purposes, qRT-PCR negative samples were treated as 1.9 log<sub>10</sub> EID<sub>50</sub>/ml. MDA:AIV<sup>-</sup>NDV<sup>+</sup> rNDV-H5(3w), progeny without AIV MDA but with NDV MDA spray-vaccinated with rNDV-H5 at 3 weeks of age; MDA:AIV<sup>+</sup>NDV<sup>+</sup> rNDV-H5(3w), progeny with AIV and NDV MDA spray-vaccinated with rNDV-H5 at 3 weeks of age; MDA:AIV<sup>-</sup>NDV<sup>+</sup> sham, progeny without AIV MDA but with NDV MDA sham-vaccinated at 3 weeks of age; MDA:AIV<sup>+</sup>NDV<sup>+</sup> sham, progeny with AIV and NDV MDA sham-vaccinated at 3 weeks of age.

**Serology.** As expected, MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny lacked AIV titers at day of hatch. Following rNDV-H5(1d) vaccine, this progeny seroconverted with low AIV titers of 4.1 and 4.3 log<sub>2</sub> GMT at 1 week, which gradually declined but were still present at 3 weeks (3.4 and 3.6 log<sub>2</sub> GMT) (Fig. 8a, Table 2). The rNDV-H5(3w) vaccine had no effect on AIV titers, as evidenced by very low levels at the time of challenge (5/10 birds with minimal detectable titers, 3 log<sub>2</sub> GMT) and lack of protection (Fig. 8a, Table 2). Progeny that received rHVT-H5(1d) vaccine had gradually increasing AIV titers that reached protective levels at challenge (Fig. 8a, Table 2). AIV titers of rHVT-H5(1d) + rNDV-H5(3w) vaccinated progeny were slightly higher than rHVT-H5(1d) vaccinated progeny at challenge, and were significantly higher at termination (Fig. 8a, Table 2).

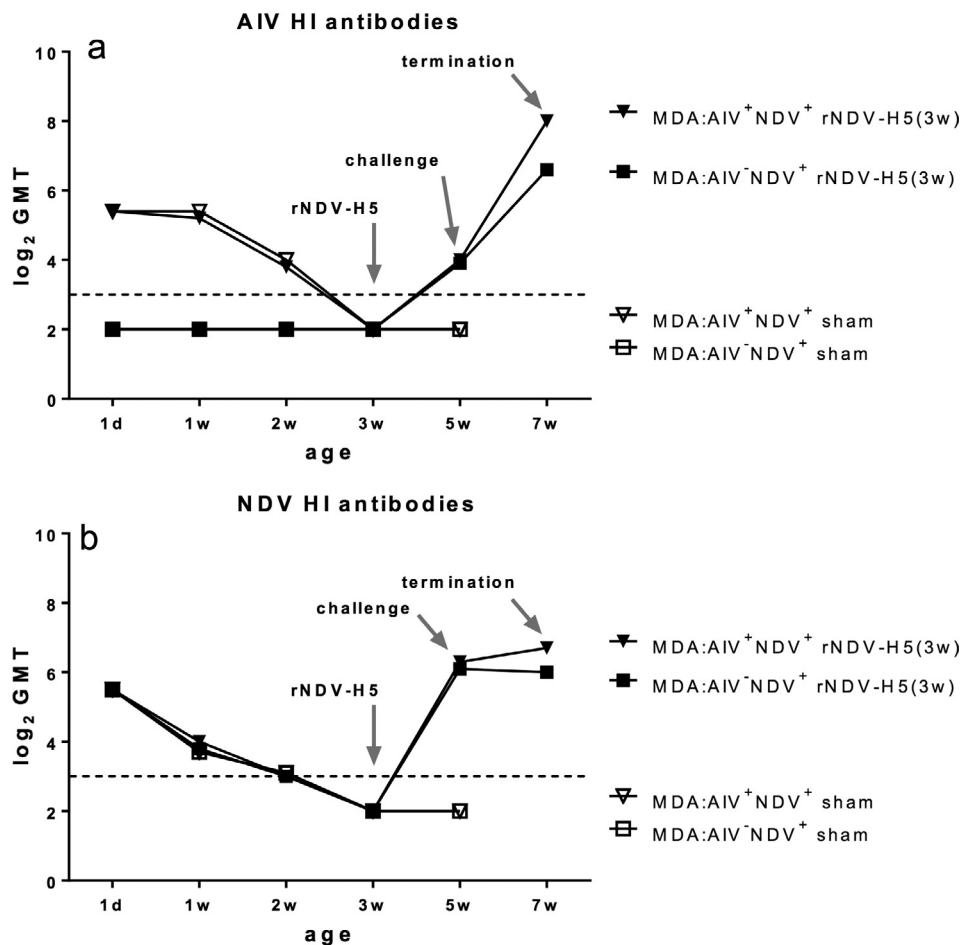
The MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny had high AIV MDA (5.5 log<sub>2</sub> GMT) at 1 day old, which gradually declined (Fig. 8b). In the presence of AIV MDA, the rNDV-H5(1d) and/or rNDV-H5(3w) vaccine had minimal effect in boosting these AIV titers, which in these groups reached very low levels by challenge (3/8 birds with detectable titers, 3 log<sub>2</sub> GMT; and 4/10 birds with detectable titers, 3.3 log<sub>2</sub> GMT) and were unable to provide clinical protection (Fig. 8a, Table 2). In the presence of AIV MDA, the rHVT-H5(1d) vaccine generated a delayed and significantly lower AIV response by the time of challenge (5.9 log<sub>2</sub> GMT) (Fig. 8b, Table 2) as compared to corresponding MDA:AIV<sup>-</sup>NDV<sup>+</sup> progeny (7.6 log<sub>2</sub> GMT) (Fig. 8a, Table 2). AIV titers of rHVT-H5(1d) + rNDV-H5(3w) vac-

inated progeny were slightly higher than rHVT-H5(1d) vaccinated progeny at challenge and termination (Fig. 8a, Table 2), suggesting a boost response from the rNDV-H5(3w) vaccination.

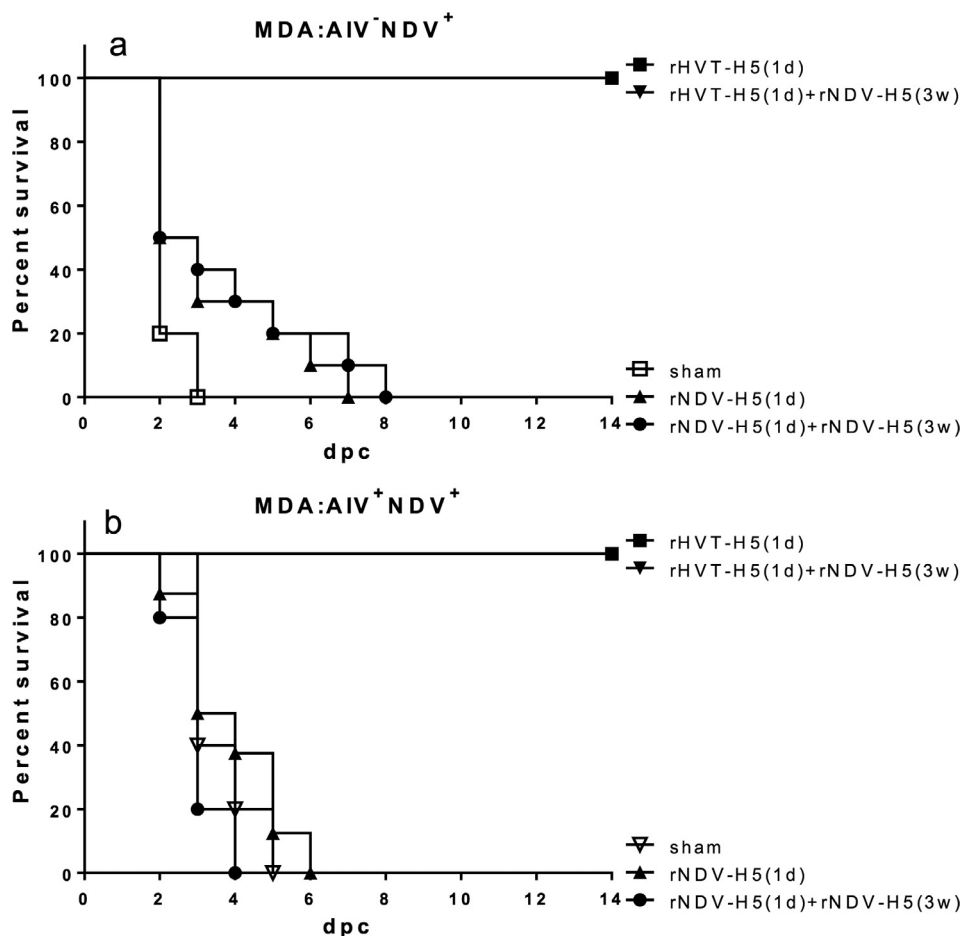
Both MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny had high NDV MDA titers (6.6 log<sub>2</sub> GMT) at 1 day old, which gradually declined but were still present at 3 weeks (3 and 3.3 log<sub>2</sub> GMT, respectively) (Fig. 8c and d). There was a correlation between ≥5 log<sub>2</sub> GMT at 1 week old and presence of antibodies at 3 weeks old ( $p = 0.0048$ ) (data not shown). The rNDV-H5(3w) vaccine following rNDV-H5(1d) generated a slight increase in NDV titers by challenge (4.1 and 4.3 log<sub>2</sub> GMT) (Fig. 8c and d, Table 2). MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny that received rHVT-H5(1d) + rNDV-H5(3w) vaccines experienced a boost in their NDV titers from 3.3 to 6.2 and 6.3 log<sub>2</sub> GMT pre-challenge, respectively (Fig. 8c and d, Table 2).

#### 4. Discussion

The continued outbreaks of HPAIV in domestic poultry worldwide emphasize the need for sustainable surveillance for variant field viruses and research to improve vaccine protection through updating seed strains. Routine vaccination may assist in reducing disease incidence and allowing the continuation of poultry production in rural settings, which maintains the livelihoods and food security of the rural poor [5]. There is growing interest for new



**Fig. 5.** Serology from progeny of Experiment 1. Follow-up HI titers for a. AIV and b. NDV antibodies from 1-day-old to 7-week-old progeny. rNDV-H5 spray vaccination at 3 weeks old, challenge with clade 2.3.4.4 Tk/MN/15 virus at 5 weeks old, and termination at 7 weeks old are indicated. Titers are expressed as log<sub>2</sub> GMT. Samples with titers below 3 log<sub>2</sub> GMT were considered negative. MDA:AIV<sup>+</sup>NDV<sup>+</sup> rNDV-H5(3w), progeny without AIV MDA but with NDV MDA spray-vaccinated with rNDV-H5 at 3 weeks of age; MDA:AIV<sup>-</sup>NDV<sup>+</sup> rNDV-H5(3w), progeny with AIV and NDV MDA spray-vaccinated with rNDV-H5 at 3 weeks of age; MDA:AIV<sup>+</sup>NDV<sup>+</sup> sham, progeny without AIV MDA but with NDV MDA sham-vaccinated at 3 weeks of age; MDA:AIV<sup>-</sup>NDV<sup>+</sup> sham, progeny with AIV and NDV MDA sham-vaccinated at 3 weeks of age.



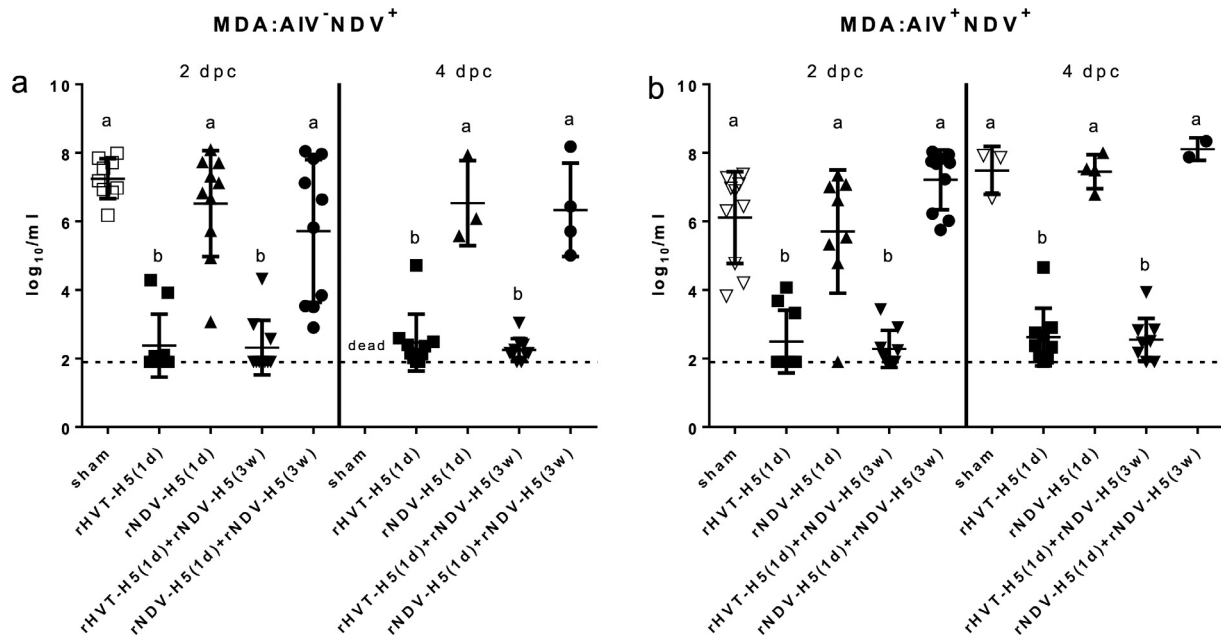
**Fig. 6.** Survival curves of Experiment 2. a. MDA:AIV<sup>-</sup>NDV<sup>+</sup> group (progeny without AIV MDA but with NDV MDA) and b. MDA:AIV<sup>+</sup>NDV<sup>+</sup> group (progeny with AIV and NDV MDA). rHVT-H5(1d), progeny subcutaneously vaccinated with rHVT-H5 at 1 day of age; rHVT-H5(1d) + rNDV-H5(3w), progeny subcutaneously vaccinated with rHVT-H5 at 1 day of age and spray-vaccinated with rNDV-H5 at 3 weeks of age; rNDV-H5(1d), progeny spray-vaccinated with rNDV-H5 at 1 day of age; rNDV-H5(1d) + rNDV-H5(3w), progeny spray-vaccinated with rNDV-H5 at 1 day of age and at 3 weeks of age; sham, progeny sham-vaccinated at 1 day of age and 3 weeks of age.

vaccines and vaccination programs using recombinant vector vaccines that can control multiple diseases at the same time, overcome MDA interference, and be mass-applied in the hatchery or later on the farm. In this study, we assessed the effectiveness of a vaccination program utilizing spray-applied rNDV-H5 vector vaccine and subcutaneous rHVT-H5 vector vaccine in commercial broilers with MDA to protect against homologous HPAIV challenge.

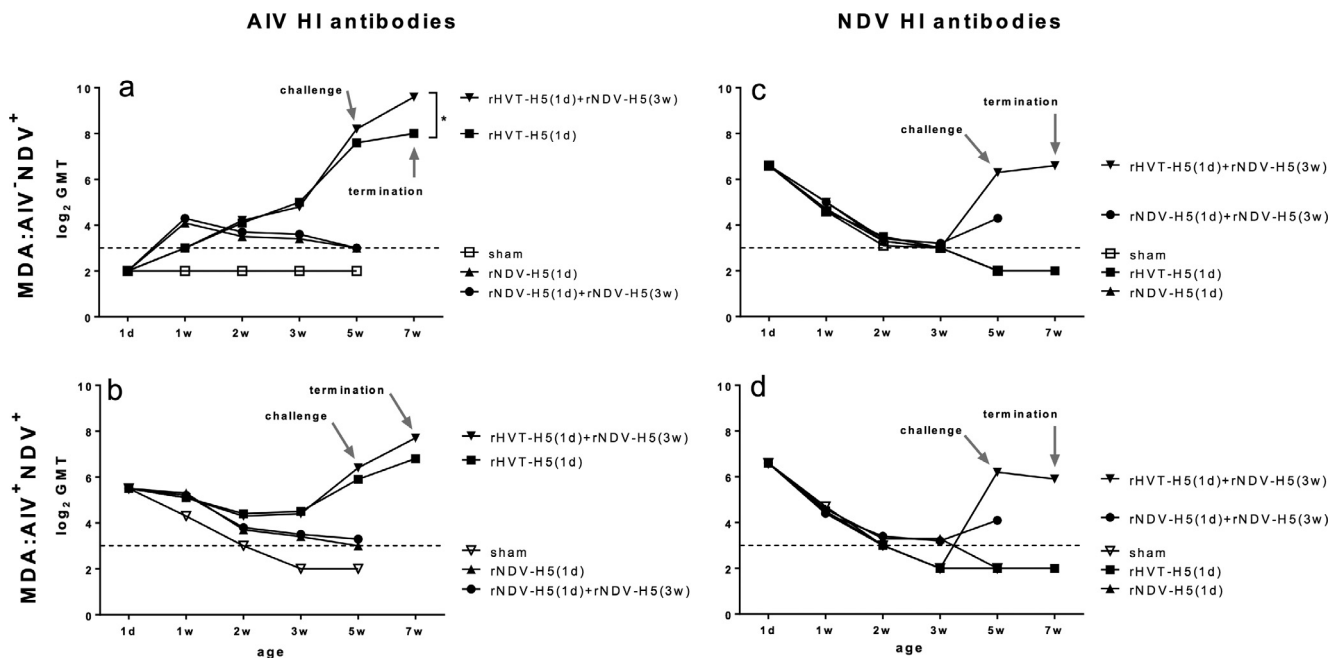
High AIV and/or NDV antibody titers were obtained in sera of hyper-immunized broiler breeders (Fig. 2) and were comparable to titers in other maternal immunity studies and in countries where vaccination to both diseases is implemented [17,24,30,31,41,43,57,58]. Antibody titers in sera of 1-day-old progeny corresponded to 68–90% of antibody titers in sera of breeders and 64–76% of antibody titers in yolk. This suggests that egg yolk samples could be used for inference of AIV and NDV MDA HI titers in the progeny, as previously suggested [58–61]. In Experiment 1, both AIV and NDV MDA completely declined to non-detectable titers by 3 weeks post-hatch. Because of the lack of MDA interference, the rNDV-H5(3w) spray-vaccine was able to replicate and elicit high and protective antibody titers (4 log<sub>2</sub> GMT AIV and 6 log<sub>2</sub> GMT NDV), leading to 70% and 78% survival and 3 log<sub>10</sub> average reduction in virus shed following homologous HPAIV challenge. Although these are sub-optimal protection results, they hint at the potential to use rNDV-H5 spray-vaccination not only at the hatchery, as described in prior studies [33,62], but also on the farm in offspring with extremely low or no AIV and/or NDV MDA. Further research is needed to fully optimize spray-vaccine of rNDV-

H5 on the farm by increasing vaccine dose, better timing vaccine administration, or testing other mass-applied delivery methods. A recent efficacy study using the same rNDV-H5 vaccine but in a different chicken model (i.e. SPF White Leghorn chickens without MDA) produced clinical protection against Tk/MN/15 challenge and reduction of virus shedding when the vaccine was administered by the intramuscular (100% survival) and spray (90% survival) routes [42]. In our study, the absence of MDA at the time of vaccination denotes a window of vulnerability to circulating HPAIV and NDV and, in an attempt to provide protection in the young susceptible broiler population, an earlier rNDV-H5 vaccination would be needed and would require overcoming possible AIV and NDV MDA interference. As expected by the lack of MDA at the time of challenge in sham-vaccinated progeny, 100% mortality and high shed virus titers were observed irrespective of AIV MDA status at the time of hatch. Whether an earlier challenge at 2 weeks (when AIV MDA were still present) instead of 5 weeks of age would have had a different survival outcome and replication efficiency in sham-vaccinated progeny remains to be determined.

In Experiment 2, our goal was to assess how prime-boost vaccination protocols using rHVT-H5 (subcutaneously at 1 day) and rNDV-H5 (spray at 1 day, 3 weeks, or both) vaccines can overcome MDA interference and provide better protection against homologous HPAIV challenge. The rHVT-H5(1d), irrespective of rNDV-H5 (3w) boost, conferred 100% clinical protection and significantly reduced virus shedding titers and number of birds shedding in both MDA:AIV<sup>-</sup>NDV<sup>+</sup> and MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny groups. In con-



**Fig. 7.** Scatter plot of oropharyngeal shedding from progeny of Experiment 2. a. MDA:AIV<sup>-</sup>NDV<sup>+</sup> group (progeny without AIV MDA but with NDV MDA) and b. MDA:AIV<sup>+</sup>NDV<sup>+</sup> group (progeny with AIV and NDV MDA). Shedding titers are expressed as  $\log_{10}$  with error bars. The limit of detection was 2.0  $\log_{10}$  EID<sub>50</sub>/ml; for statistical purposes, qRRT-PCR negative samples were treated as 1.9  $\log_{10}$  EID<sub>50</sub>/ml. rHVT-H5(1d), progeny subcutaneously vaccinated with rHVT-H5 at 1 day of age; rHVT-H5(1d) + rNDV-H5(3w), progeny subcutaneously vaccinated with rHVT-H5 at 1 day of age and spray-vaccinated with rNDV-H5 at 3 weeks of age; rNDV-H5(1d), progeny spray-vaccinated with rNDV-H5 at 1 day of age; rNDV-H5(1d) + rNDV-H5(3w), progeny spray-vaccinated with rNDV-H5 at 1 day of age and at 3 weeks of age; sham, progeny sham-vaccinated at 1 day of age and 3 weeks of age.



**Fig. 8.** Serology from progeny of Experiment 2. Follow-up HI titers for AIV and NDV antibodies from 1-day-old to 7-week-old progeny. Challenge with clade 2.3.4.4 Tk/MN/15 virus at 5 weeks old and termination at 7 weeks old are indicated. Titers are expressed as  $\log_2$  GMT. Samples with titers below 3  $\log_2$  GMT were considered negative. MDA: AIV<sup>-</sup>NDV<sup>+</sup>, progeny without AIV MDA but with NDV MDA; MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny with AIV and NDV MDA; rHVT-H5(1d), progeny subcutaneously vaccinated with rHVT-H5 at 1 day of age; rHVT-H5(1d) + rNDV-H5(3w), progeny subcutaneously vaccinated with rHVT-H5 at 1 day of age and spray-vaccinated with rNDV-H5 at 3 weeks of age; rNDV-H5(1d), progeny spray-vaccinated with rNDV-H5 at 1 day of age; rNDV-H5(1d) + rNDV-H5(3w), progeny spray-vaccinated with rNDV-H5 at 1 day of age and at 3 weeks of age; sham, progeny sham-vaccinated at 1 day of age and 3 weeks of age.

trast, progeny that received spray rNDV-H5(1d) and/or rNDV-H5(3w) vaccine had 100% mortality irrespective of the MDA group. Our results demonstrate that rHVT-H5(1d) could overcome AIV MDA present at hatch and produce a protective immune response against HPAIV. In contrast, the replication and H5-insert expres-

sion of rNDV-H5 (both at 1 day and at 3 weeks) were negatively impacted by AIV and/or NDV MDA. These results align with previous studies which indicate that rHVT-H5 would be better as a primary single dose vaccine or priming vector [39,63] in a prime-boost regime than rNDV-H5 [24,28,31,33,43,63] for progeny with

NDV and/or H5 AIV MDA. It is worth emphasizing that the presence of H5 AIV MDA in MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny in our study seemed to interfere to a certain degree with rHVT-H5(1d), since it generated a significantly lower (albeit still protective) AIV response as compared to corresponding MDA:AIV<sup>+</sup>NDV<sup>+</sup> progeny. Besides, because breeders received cell-associated HVT *in ovo* vaccination that induces protection through cell-mediated immunity, any antibodies that passed to the chick failed to prevent rHVT-H5 expression and immunity to AIV.

Differences in protection conferred by rNDV-H5 vaccine between Experiments 1 and 2 could be explained by interference of residual MDA antibodies at the time of rNDV-H5 vaccination in Experiment 2, either due to higher antibody titers in hens and progeny, and/or due to interference by prime vaccination at day of age. While progeny in Experiment 1 had no measurable AIV or NDV MDA at 3 weeks (i.e. time of vaccination), progeny in Experiment 2 had both AIV and NDV MDA and/or titers elicited by priming vaccination at 1 day, negatively impacting rNDV-H5 replication at 3 weeks (i.e. time of boost) and resulting in no or minimal clinical protection and high challenge virus shedding. The presence of  $\geq 5$  log<sub>2</sub> GMT of AIV MDA at 1 week of age correlated with the presence of MDA at 3 weeks of age, which predicted interference with rNDV-H5 vaccination. Therefore, measuring the level of MDA at a very young age in offspring obtained from hyper-immunized breeders could be a strategy to predict the optimal timing for vaccination when rNDV-H5 vaccine is used, as has been suggested for vaccination against IBDV [64,65].

The AIV titers of rHVT-H5(1d) + rNDV-H5(3w) vaccinated progeny were slightly higher than rHVT-H5(1d) vaccinated progeny at challenge, and significantly higher at termination. It is therefore hypothesized that the primary H5-antibody response with rHVT-H5(1d) was amplified by memory B cells after rNDV-H5(3w) boost. Nonetheless, immunologic protection mechanisms other than the systemic humoral response, such as local mucosal and cellular immunity, could be implicated. A single dose of rHVT-H5 at 1 day has been recommended to protect for the short 7-week life of a broiler [36]. However, a low cost mass-applied booster vaccination may be needed to provide protection in longer-lived poultry. Based on our study, a higher dose of rNDV-H5(3w) should be examined as a possible strategy to overcome the inhibitory effect of low AIV and/or NDV MDA titers and increase H5 expression, as has been proposed [43].

The use of commercial broilers in the current study allowed for a more practical interpretation of effectiveness for virus vectored vaccine field application than the use of SPF White Leghorn chickens. Indeed, broilers in the field are constantly exposed to a wider range of pathogens (i.e. not SPF) and vaccines (i.e. pre-existing immunity) and are genetically programmed for a lower primary humoral response to vaccines [66]. Furthermore, a protective immune response in the field is more difficult to achieve than in an experimental setting and can be hampered due to improper use of vaccines, poor management, or co-infection with other pathogens. These factors may significantly reduce the success rate of vaccination programs under field conditions as compared to single vaccination of SPF White Leghorn chickens in an experimental setting, thus requiring multiple booster vaccinations [67,68].

In conclusion, the present study contributes to a better understanding of the practical use of rHVT-H5 and rNDV-H5 vector vaccines in commercial broilers within H5 AIV-enzootic countries which have H5 AIV and/or NDV MDA. We demonstrated that optimized spray-application of rNDV-H5 vaccine could be feasible on the farm in the absence of AIV and NDV MDA at 3 weeks of age, and that 1-day application of rHVT-H5 is able to overcome the neutralizing effect of AIV MDA present at 1 day of age, therefore making rHVT-H5 a better priming vector than rNDV-H5 for progeny with AIV and NDV MDA. Certain questions remain unanswered

and open new lines of research: (i) would rNDV-H5 vaccinated progeny have been protected against velogenic NDV challenge; (ii) would higher doses of rNDV-H5 vaccine be able to overcome low titers of AIV and NDV MDA and achieve satisfactory protection against homologous H5 HPAIV challenge; and (iii) will chimeric rNDV-based vector vaccines [69] or other APMV-based vector vaccines (APMV-2 to -13) be able to overcome NDV MDA?

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## Conflict of interest

The authors declare that they have no conflict of interest.

## References

- [1] Suarez DL, Lee CW, Swayne DE. Avian influenza vaccination in North America: strategies and difficulties. *Dev Biol (Basel)* 2006;124:117–24.
- [2] Sims LD, Swayne DE. Avian influenza control strategies. In: Swayne DE, editor. *Animal Influenza*. Ames, IA: Blackwell Publishing; 2016. p. 363–77.
- [3] Xu X, Subbarao Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/Goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. *Virology* 1999;261(1):15–9.
- [4] Lee DH, Bertran K, Kwon JH, Swayne DE. Evolution global spread, and pathogenicity of highly pathogenic avian influenza H5Nx clade 2.3.4.4. *J Vet Sci* 2017;18(S1):269–80.
- [5] Swayne DE, Pavade G, Hamilton K, Vallat B, Miyagishima K. Assessment of national strategies for control of high-pathogenicity avian influenza and low-pathogenicity notifiable avian influenza in poultry, with emphasis on vaccines and vaccination. *Rev Sci Tech Off Int Epiz* 2011;30:839–70.
- [6] Miller PJ, Koch G. Newcastle disease. In: Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair VL, editors. *Diseases of poultry*. Hoboken, New Jersey: Wiley-Blackwell; 2013. p. 89–138.
- [7] Afonso CL, Amarasinghe GK, Banyai K, Bao Y, Basler CF, Bavari S, et al. Taxonomy of the order Mononegavirales: update 2016. *Arch Virol* 2016;161(8):2351–60.
- [8] Amarasinghe GK, Bao Y, Basler CF, Bavari S, Beer M, Bejerman N, et al. Taxonomy of the order Mononegavirales: update 2017. *Arch Virol* 2017;162(8):2493–504.
- [9] Dimitrov KM, Ramey AM, Qiu X, Bahl J, Afonso CL. Temporal, geographic, and host distribution of avian paramyxovirus 1 (Newcastle disease virus). *Infect Genet Evol* 2016;39:22–34.
- [10] Schat KA, Nair VL. Neoplastic diseases Marek's disease. In: Swayne DE, Glisson JR, McDougald LR, Nolan LK, Suarez DL, Nair VL, editors. *Diseases of poultry*. Wiley; 2013. p. 513–674.
- [11] Reddy SM, Izumiya Y, Lupiani B. Marek's disease vaccines: Current status, and strategies for improvement and development of vector vaccines. *Vet Microbiol* 2017;206:113–20.
- [12] Senne DA, King DJ, Kapczynski DR. Control of Newcastle disease by vaccination. *Dev Biol (Basel)* 2004;119:165–70.
- [13] Mayers J, Mansfield KL, Brown IH. The role of vaccination in risk mitigation and control of Newcastle disease in poultry. *Vaccine* 2017;35(44):5974–80.
- [14] Sakaguchi M, Nakamura H, Sonoda K, Okamura H, Yokogawa K, Matsuo K, et al. Protection of chickens with or without maternal antibodies against both Marek's and Newcastle diseases by one-time vaccination with recombinant vaccine of Marek's disease virus type 1. *Vaccine* 1998;16(5):472–9.
- [15] Lee CW, Senne DA, Suarez DL. Effect of vaccine use in the evolution of Mexican lineage H5N2 avian influenza virus. *J Virol* 2004;78:8372–81.
- [16] Rauw F, Gardin Y, Palya V, van Borm S, Gonze M, Lemaire S, et al. Humoral, cell-mediated and mucosal immunity induced by oculo-nasal vaccination of

- one-day-old SPF and conventional layer chicks with two different live Newcastle disease vaccines. *Vaccine* 2009;27(27):3631–42.
- [17] Maas R, Rosema S, van Zoelen D, Venema S. Maternal immunity against avian influenza H5N1 in chickens: limited protection and interference with vaccine efficacy. *Avian Pathol* 2011;40(1):87–92.
  - [18] Brambell FWR. Transmission of immunity in birds. In: Neuberger A, Tatum EL, editors. *Transmission of passive immunity from mother to young*, vol. 18. New York, NY: Elsevier; 1970. p. 20–41.
  - [19] Hamal KR, Burgess SC, Pevzner IY, Erf GF. Maternal antibody transfer from dams to their egg yolks, egg whites, and chicks in meat lines of chickens. *Poult Sci* 2006;85(8):1364–72.
  - [20] Mast J, Goddeeris BM. Development of immunocompetence of broiler chickens. *Vet Immunol Immunopathol* 1999;70(3–4):245–56.
  - [21] Al-Natour MQ, Ward LA, Saif YM, Stewart-Brown B, Keck LD. Effect of different levels of maternally derived antibodies on protection against infectious bursal disease virus. *Avian Dis* 2004;48(1):177–82.
  - [22] Naqi SA, Marquez B, Sahin N. Maternal antibody and its effect on infectious bursal disease immunization. *Avian Dis* 1983;27(3):623–31.
  - [23] van Eck JH, van Wiltenburg N, Jaspers D. An Ulster 2C strain-derived Newcastle disease vaccine: efficacy and excretion in maternally immune chickens. *Avian Pathol* 1991;20(3):481–95.
  - [24] Kapczynski DR, King DJ. Protection of chickens against overt clinical disease and determination of viral shedding following vaccination with commercially available Newcastle disease virus vaccines upon challenge with highly virulent virus from the California 2002 exotic Newcastle disease outbreak. *Vaccine* 2005;23(26):3424–33.
  - [25] Kim JK, Kayali G, Walker D, Forrest HL, Ellebedy AH, Griffin YS, et al. Puzzling inefficiency of H5N1 influenza vaccines in Egyptian poultry. *Proc Natl Acad Sci USA* 2010;107(24):11044–9.
  - [26] De Vriese J, Steensels M, Palya V, Gardin Y, Dorsey KM, Lambrecht B, et al. Passive protection afforded by maternally-derived antibodies in chickens and the antibodies' interference with the protection elicited by avian influenza-inactivated vaccines in progeny. *Avian Dis* 2010;54(1 Suppl):246–52.
  - [27] Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM. Influence of maternal immunity on vaccine efficacy and susceptibility of one day old chicks against Egyptian highly pathogenic avian influenza H5N1. *Vet Microbiol* 2012;155(1):13–20.
  - [28] Faulkner OB, Estevez C, Yu Q, Suarez DL. Passive antibody transfer in chickens to model maternal antibody after avian influenza vaccination. *Vet Immunol Immunopathol* 2013;152(3–4):341–7.
  - [29] Forrest HL, Garcia A, Danner A, Seiler JP, Friedman K, Webster RG, et al. Effect of passive immunization on immunogenicity and protective efficacy of vaccination against a Mexican low-pathogenic avian H5N2 influenza virus. *Influenza Other Respir Viruses* 2013;7(6):1194–201.
  - [30] Richard-Mazet A, Goutebroze S, Le Gros FX, Swayne DE, Bublot M. Immunogenicity and efficacy of fowlpox-vectored and inactivated avian influenza vaccines alone or in a prime-boost schedule in chickens with maternal antibodies. *Vet Res* 2014;45:107. 014-0107-6.
  - [31] Lardinois A, Vandersleyen O, Steensels M, Desloges N, Mast J, van den Berg T, et al. Stronger interference of avian influenza virus-specific than newcastle disease virus-specific maternally derived antibodies with a recombinant NDV-H5 vaccine. *Avian Dis* 2016;60(1 Suppl):191–201.
  - [32] Domenech J, Dauphin G, Rushton J, McGrane J, Lubroth J, Tripodi A, et al. Experiences with vaccination in countries endemically infected with highly pathogenic avian influenza: the Food and Agriculture Organization perspective. *Rev Sci Tech* 2009;28(1):293–305.
  - [33] Spackman E, Swayne DE. Vaccination of gallinaceous poultry for H5N1 highly pathogenic avian influenza: current questions and new technology. *Virus Res* 2013;178(1):121–32.
  - [34] Yewdell JW, Norburch CC, Bennink JR. Mechanisms of exogenous antigen presentation by MHC class I molecules in vitro and in vivo: implications for generating CD8+ T cell responses to infectious agents, tumors, transplants, and vaccines. *Adv Immunol* 1999;73:1–77.
  - [35] Swayne DE, Beck JR, Kinney N. Failure of a recombinant fowl poxvirus vaccine containing an avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox vaccine. *Avian Dis* 2000;44(1):132–7.
  - [36] Suarez DL, Pantin-Jackwood MJ. Recombinant viral-vectored vaccines for the control of avian influenza in poultry. *Vet Microbiol* 2017;206:144–51.
  - [37] Kapczynski DR, Esaki M, Jackwood MW, Dorsey KM. Vaccination of SPF chickens with a recombinant HVT expressing the HA from H5N1 highly pathogenic avian influenza protects against lethal challenge. In: David F, editor. *Proceedings of the 59th western poultry disease conference*. Vancouver, Canada; 2010. p. 124.
  - [38] Rauw F, Palya V, Van Borm S, Welby S, Tatar-Kis T, Gardin Y, et al. Further evidence of antigenic drift and protective efficacy afforded by a recombinant HVT-H5 vaccine against challenge with two antigenically divergent Egyptian clade 2.2.1 HPAI H5N1 strains. *Vaccine* 2011;29(14):2590–600.
  - [39] Rauw F, Palya V, Gardin Y, Tatar-Kis T, Dorsey KM, Lambrecht B, et al. Efficacy of rHVT-AI vector vaccine in broilers with passive immunity against challenge with two antigenically divergent Egyptian clade 2.2.1 HPAI H5N1 strains. *Avian Dis* 2012;56(4 Suppl):913–22.
  - [40] Soejoedono RD, Murtini S, Palya V, Felfoldi B, Mato T, Gardin Y. Efficacy of a recombinant HVT-H5 vaccine against challenge with two genetically divergent Indonesian HPAI H5N1 strains. *Avian Dis* 2012;56(4 Suppl):923–7.
  - [41] Kilany W, Dauphin G, Selim A, Tripodi A, Samy M, Sobhy H, et al. Protection conferred by recombinant turkey herpesvirus avian influenza (rHVT-H5) vaccine in the rearing period in two commercial layer chicken breeds in Egypt. *Avian Pathol* 2014;43(6):514–23.
  - [42] Ma J, Lee J, Liu H, Mena I, Davis AS, Sunwoo SY, et al. Newcastle disease virus-based H5 influenza vaccine protects chickens from lethal challenge with a highly pathogenic H5N2 avian influenza virus. *NPJ Vacc* 2017;2:33. 017-0034-4. eCollection 2017.
  - [43] Sarfati-Mizrahi D, Lozano-Dubernard B, Soto-Priante E, Castro-Peralta F, Flores-Castro R, Loza-Rubio E, et al. Protective dose of a recombinant Newcastle disease LaSota-avian influenza virus H5 vaccine against H5N2 highly pathogenic avian influenza virus and velogenic viscerotropic Newcastle disease virus in broilers with high maternal antibody levels. *Avian Dis* 2010;54(1 Suppl):239–41.
  - [44] DiNapoli JM, Yang L, Suguitan Jr A, Elankumaran S, Dorward DW, Murphy BR, et al. Immunization of primates with a Newcastle disease virus-vectored vaccine via the respiratory tract induces a high titer of serum neutralizing antibodies against highly pathogenic avian influenza virus. *J Virol* 2007;81(21):11560–8.
  - [45] Dimitrov KM, Afonso CL, Yu Q, Miller PJ. Newcastle disease vaccines-A solved problem or a continuous challenge? *Vet Microbiol* 2017;206:126–36.
  - [46] Stone HD, Brugh M, Beard CW. Influence of formulation on the efficacy of experimental oil-emulsion Newcastle disease vaccines. *Avian Dis* 1983;27(3):688–97.
  - [47] Stone HD. Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. *Avian Dis* 1987;31(3):483–90.
  - [48] Swayne DE, Beck JR, Garcia M, Stone HD. Influence of virus strain and antigen mass on efficacy of H5 avian influenza inactivated vaccines. *Avian Pathol* 1999;28:245–55.
  - [49] Bertran K, Moresco K, Swayne DE. Impact of vaccination on infection with Vietnam H5N1 high pathogenicity avian influenza virus in hens and the eggs they lay. *Vaccine* 2015;33(11):1324–30.
  - [50] DeJesus E, Costa-Hurtado M, Smith D, Lee DH, Spackman E, Kapczynski DR, et al. Changes in adaptation of H5N2 highly pathogenic avian influenza H5 clade 2.3.4.4 viruses in chickens and mallards. *Virology* 2016;499:52–64.
  - [51] Bertran K, Lee DH, Balzli C, Pantin-Jackwood MJ, Spackman E, Swayne DE. Age is not a determinant factor in susceptibility of broilers to H5N2 clade 2.3.4.4 high pathogenicity avian influenza virus. *Vet Res* 2016;47(1):116.
  - [52] Spackman E, Killian ML. Avian influenza virus isolation, propagation, and titration in embryonated chicken eggs. *Methods Mol Biol* 2014;1161:125–40.
  - [53] Pedersen JC. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. In: Spackman E, editor. *Animal influenza virus*. Springer; 2014. p. 11–26.
  - [54] Abbas MA, Spackman E, Fouchier R, Smith D, Ahmed Z, Siddique N, et al. H7 avian influenza virus vaccines protect chickens against challenge with antigenically diverse isolates. *Vaccine* 2011;29:7424–9.
  - [55] Spackman E, Senne DA, Myers TJ, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. *J Clin Microbiol* 2002;40(9):3256–60.
  - [56] Slomka MJ, Densham AL, Coward VJ, Essen S, Brookes SM, Irvine RM, et al. Real time reverse transcription (RRT)-polymerase chain reaction (PCR) methods for detection of pandemic (H1N1) 2009 influenza virus and European swine influenza A virus infections in pigs. *Influenza Other Respir Viruses* 2010;4(5):277–93.
  - [57] Lee DH, Kwon JS, Lee HJ, Lee YN, Hur W, Hong YH, et al. Inactivated H9N2 avian influenza virus vaccine with gel-primed and mineral oil-boosted regimen could produce improved immune response in broiler breeders. *Poult Sci* 2011;90(5):1020–2.
  - [58] Abdelwhab EM, Grund C, Aly MM, Beer M, Harder TC, Hafez HM. Benefits and limits of egg yolk vs. serum samples for avian influenza virus serosurveillance. *Avian Dis* 2016;60(2):496–9.
  - [59] Beck JR, Swayne DE, Davison S, Casavant S, Gutierrez C. Validation of egg yolk antibody testing as a method to determine influenza status in white leghorn hens. *Avian Dis* 2003;47(3 Suppl):1196–9.
  - [60] Trampel DW, Zhou EM, Yoon KJ, Koehler KJ. Detection of antibodies in serum and egg yolk following infection of chickens with an H6N2 avian influenza virus. *J Vet Diagn Invest* 2006;18(5):437–42.
  - [61] Sa e Silva M, Swayne DE. Serum and egg yolk antibody detection in chickens infected with low pathogenicity avian influenza virus. *Avian Dis* 2012;56(3):601–4.
  - [62] Swayne DE, Kapczynski D. Strategies and challenges for eliciting immunity against avian influenza virus in birds. *Immunol Rev* 2008;225:314–31.
  - [63] Spackman E, Pantin-Jackwood MJ. Practical aspects of vaccination of poultry against avian influenza virus. *Vet J* 2014;202(3):408–15.
  - [64] Block H, Meyer-Block K, Rebeski DE, Scharr H, de Wit S, Rohn K, et al. A field study on the significance of vaccination against infectious bursal disease virus (IBDV) at the optimal time point in broiler flocks with maternally derived IBDV antibodies. *Avian Pathol* 2007;36(5):401–9.
  - [65] De Herdt P, Jagt E, Paul G, Van Colen S, Renard R, Destrooper C, et al. Evaluation of the enzyme-linked immunosorbent assay for the detection of antibodies against infectious bursal disease virus (IBDV) and the estimation of the optimal age for IBDV vaccination in broilers. *Avian Pathol* 2005;34(6):501–4.

- [66] Koenen ME, Boonstra-Blom AG, Jeurissen SH. Immunological differences between layer- and broiler-type chickens. *Vet Immunol Immunopathol* 2002;89(1–2):47–56.
- [67] Eggert D, Swayne DE. Single vaccination provides limited protection to ducks and geese against H5N1 high pathogenicity avian influenza virus. *Avian Dis* 2010;54:1224–9.
- [68] Swayne DE, Spackman E, Pantin-Jackwood M. Success factors for avian influenza vaccine use in poultry and potential impact at the wild bird-agricultural interface. *Ecohealth* 2014;11(1):94–108.
- [69] Steglich C, Grund C, Ramp K, Breithaupt A, Hoper D, Keil G, et al. Chimeric newcastle disease virus protects chickens against avian influenza in the presence of maternally derived NDV immunity. *PLoS One* 2013;8(9):e72530.